Study Details

Test No. 2016366
Product Name PI 1525

Cas-No:	EC-No:	Chemical Name:
1897392-68-5		4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4R,5S,7R,7aR)-rel-

Product code	
Product Name	PI 1525
Test code	PI 1525
Purity	95,3 (if 0,0 then see remarks)
Batch No.	Ho154262-MM+0.1% Vit. E.
Study code	1783501
Institute Name	Envigo CRS GmbH (former HARLAN)
Description	In Vitro Skin irritation: Reconstructed Human Epidermis Test Method, OECD 439, EU B.46 bis
Final Report date	30.01.2017
Results	skin irritant
Reliability	Rel 1
GLP	YES
Remark	Human Skin Model test with 30µL test item undiluted, tissue viability was 27.9 % (threshold for classification <50%) Rel. 1: according to OECD 439 (2015), EU B.46 (Commission Regulation 440/2008, 1st ATP 2009), UN GHS (6rd revision 2015) and GLP

Report

MIXTURE OF 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-, (3AR,4R,5S,7R,7AR)-REL- AND 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-,(3AR,4S,5R,7S,7AR)-REL-: *In vitro* Skin Irritation Test: Human Skin Model Test

Envigo Study Number:	1783501
Sponsor Name:	
Version ID:	Final – 1st Original of 3
Issue Date:	30 January 2017
Study Director:	Dr. Markus Roth
Testing Facility:	Envigo CRS GmbH In den Leppsteinswiesen 19 64380 Rossdorf Germany

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DEFINITIONS AND ABBREVIATIONS

ATLA	Alternative to Laboratory Animals
ATP	Adaption to Technical Process
CLP	Classification, Labelling and Packaging Regulation
DMEM	Dulbecco's Minimum Essential Medium
DPBS	Dulbecco's Phosphate Buffered Saline
EC	European Commission
ECVAM	European Centre for the Validation of Alternative Methods
EEC	European Economic Community
EU	European Union
GHS	Globally Harmonised System
GLP	Good Laboratory Practice
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazoliumbromide
OD	Optical Density
OECD	Organisation for Economic Co-Operation and Development
QA	Quality Assurance
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RH	Relative Humidity
SLS	Sodium Lauryl Sulphate
TG	Test Guideline
UN	United Nations

COMPLIANCE WITH GOOD LABORATORY PRACTICE

MIXTURE OF 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-, (3AR,4R,5S,7R,7AR)-REL- AND 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-,(3AR,4S,5R,7S,7AR)-REL-: *In vitro* Skin Irritation Test: Human Skin Model Test

The study described in this report was conducted in compliance with the following Good Laboratory Practice standards and I consider the data generated to be valid.

- "Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1) in its currently valid version
- OECD Principles of Good Laboratory Practice (as revised in 1997), ENV/MC/CHEM(98)17
- EC Commission Directive 2004/10/EC

These principles of Good Laboratory Practice are accepted by the members of the OECD Mutual Acceptance of Data including the European Community/United States of America and Japan.

30 January 2017

Dr.Markus Roth
Study Director
Envigo CRS GmbH

Date

QUALITY ASSURANCE STATEMENT

MIXTURE OF 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-, (3AR,4R,5S,7R,7AR)-REL- AND 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-,(3AR,4S,5R,7S,7AR)-REL-: *In vitro* Skin Irritation Test: Human Skin Model Test

Study based activities at the Test Facility Envigo CRS GmbH, Rossdorf, were audited and inspected. The details of these audits and inspections are given below.

Type of Inspection	Date(s) of Inspection	Date Reporting to Study Director, Test Facility Management
Study Plan Verification	12 July 2016	12 July 2016
Study Plan Amendment 1 Verification	20 December 2016	20 December 2016
Process – based Test System Preparation and Application	13 July 2016	13 July 2016
Report Audit	14 October 2016	14 October 2016

General facilities and activities where this study was conducted were inspected on an annual basis and results are reported to the relevant responsible person and Management.

Quality Assurance

S. Ebert

30 January 2017

Sabine Ebert

Date

Quality Assurance Auditor
Envigo CRS GmbH

1 SUMMARY

This *in vitro* study was performed to assess the irritation potential of MIXTURE OF 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-, (3AR,4R,5S,7R,7AR)-REL- AND 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-,(3AR,4S,5R,7S,7AR)-REL- by means of the Human Skin Model Test.

The test item did not reduce MTT (test for direct MTT reduction), and it did not change colour when mixed with deionised water (test for colour interference). Also its intrinsic colour was not intensive. Consequently, additional tests with freeze-killed or viable tissues were not necessary.

Each three tissues of the human skin model EpiDerm™ were treated with the test item, the negative or the positive control for 60 minutes.

30 µL of the test item were applied to each tissue, and spread to match the surface of the tissue.

30 µL of either the negative control (DPBS) or the positive control (5% SLS) were applied to each tissue.

After treatment with the negative control the absorbance values were well within the required range of the acceptability criterion of mean OD ≥ 0.8 and ≤ 2.8 for the 60 minutes treatment interval, thus assuring the quality of the tissues.

Treatment with the positive control induced a sufficient decrease in the relative absorbance as compared to the negative control for the 60 minutes treatment interval, and thus assuring the validity of the test system.

After treatment with the test item MIXTURE OF 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-, (3AR,4R,5S,7R,7AR)-REL- AND 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-,(3AR,4S,5R,7S,7AR)-REL- the mean relative absorbance value decreased relevantly to 27.9% compared to the relative absorbance value of the negative control. This value is below the threshold for irritancy of ≤ 50%. Therefore, the test item is considered to possess an irritant potential.

In conclusion, it can be stated that in this study and under the experimental conditions reported, MIXTURE OF 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-, (3AR,4R,5S,7R,7AR)-REL- AND 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-,(3AR,4S,5R,7S,7AR)-REL- **is irritant** to skin.

2 INTRODUCTION AND PURPOSE

Dermal irritation is generally defined as "the production of reversible inflammatory changes in the skin". The potential for chemical induced skin irritation is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals. It is usually determined *in vivo* in the Draize rabbit skin irritation test as described in OECD guideline 404. Because systemic reactions play a minor role in modulating local skin toxicity potential of chemicals, skin irritation potential may be predicted by *in vitro* systems, provided they are sufficiently complex to mimic human skin barrier and cell reactivity. In an international prevalidation study performed by ECVAM, the in vitro skin irritation test using the human skin model EpiDerm™ and EpiSkin™ and measurement of cell viability by dehydrogenase conversion of MTT into a blue formazan salt have turned out as a sufficiently promising predictor for skin irritancy potential.

The test consists of a topical exposure of the neat test item to a human reconstructed epidermis model followed by a cell viability test. Cell viability is measured by dehydrogenase conversion of MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazoliumbromide], in cell mitochondria, into a blue formazan salt that is quantitatively measured after extraction from tissues. The percent reduction of cell viability in comparison of untreated negative controls is used to predict skin irritation potential (see OECD TG 439) and is used for the purpose of classification as irritating or non-irritating according to chemicals law (EU CLP, UN GHS). Depending on the regulatory framework and applicability of the test guideline, chemicals that produce cell viabilities above the defined threshold level, are considered non-irritants. The test chemical is considered to be irritant to skin in accordance with UN GHS and EU CLP Category 2 if the tissue viability after exposure and post-treatment incubation is less than or equal (≤) to 50%.

2.1 Study Details

Sponsor:

Study Monitor:

2.2 Study Schedule

Experimental start date**:** 21 July 2016

Experimental completion date**:** 15 August 2016

2.3 Regulatory Testing Guidelines

The study was performed in compliance with the following regulations or guidelines:

- OECD Guideline for the testing of Chemicals 439: *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* Test Method (Original Guideline adopted July 28, 2015), and as described in detail in the Protocol for: *In Vitro* EpiDerm™ Skin Irritation Test (EPI-200-SIT) for use with MatTek Corporation's Reconstructed Human Epidermal Model EpiDerm (EPI-200), Rev. 29/06/15.
- Based on a "Statement on the Scientific Validity of *In Vitro* Tests for Skin Irritation" of the European Commission (November 2008), official acceptance of the test method in the EU was achieved and implemented in EU, 2008a, Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to EC Regulation No 1907/2006 of the European Parliament and of the Council on REACH; 1st ATP 2009: EC Regulation No 761/2009 of 23 July 2009 amending, for the purpose of its ATP, EC Regulation No 440/2008 laying down test methods pursuant to EC Regulation No 1907/2006 of the European Parliament and of the Council on REACH, section B46.
- UN GHS (published 2003, last (6th) revision 2015)

3 MATERIALS AND TEST METHODS

3.1 Test Item and Supporting Information

Information as provided by the Sponsor.

Identification:	MIXTURE OF 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-, (3AR,4R,5S,7R,7AR)-REL- AND 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-,(3AR,4S,5R,7S,7AR)-REL-
Synonyms:	4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4R,5S,7R,7aR)-rel-
CAS No.:	1897392-68-5 and 132130- 08-6
Batch:	Ho 154 262 MM + 0.1% Vit. E
Purity:	95.3%, Correction for purity was not made.
Appearance:	Liquid, clear
Expiry Date:	December 2017
Storage Conditions:	At room temperature, under light protection
Stability in Solvent:	Not indicated by the Sponsor
Purpose of Use:	Industrial chemical

3.2 Study Controls

Concurrent controls were used for several Envigo CRS GmbH studies performed simultaneously.

3.2.1 Negative Control

30 µL DPBS (MatTek) were used as negative control per tissue.

3.2.2 Positive Control

30 µL of a 5% SLS solution in deionised water (MatTek) were used a positive control per tissue.

3.3 Test Item Preparation

30 µL (47 µL/cm^2 according to guideline) of the undiluted test item was dispensed directly atop the EpiDerm™ tissue and spread to match the surface of the tissue for a complete treatment time of 60 minutes.

3.4 Test System and Supporting Information

3.4.1 Epi-200- SIT Kit Components Needed for the Assay

Epi-200- PHO Kit Lot No.: 23349

1	Sealed 24-well plate	Contains 24 inserts with EpiDerm™ tissues on agarose
2	24-well plates	For MTT viability assay
4	6-well plates	For storing inserts, or for topically applying test agents
1 bottle	Serum-Free Assay Medium	DMEM-based medium
1 bottle	DPBS Rinse Solution	For rinsing the inserts in MTT assay

3.4.2 MTT-100 Assay Kit Components

1 vial, 2 mL	MTT concentrate	
1 vial, 8 mL	MTT diluent (supplemented DMEM)	For diluting MTT concentrate prior to use in the MTT assay
1 bottle	Extractant Solution (Isopropanol)	For extraction of formazan crystals

3.4.3 Cell Culture

Epi-200 SIT kits and MTT-100 assays diluent were purchased from MatTek Corporation (82105 Bratislava, Slovakia). The EpiDerm™ tissue consists of normal, human-derived epidermal keratinocytes which have been cultured to form a multilayered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multi-layered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found in vivo. The EpiDerm™ tissues (surface 0.6 cm^2) are cultured on specially prepared cell culture inserts (MILLICELLs®, 10 mm ∅).

EpiDerm™ tissues were shipped with cool packs on medium-supplemented agarose gels in a 24-well plate and reached Envigo CRS GmbH on 09 August 2016. On day of receipt the pre-incubation phase of the EpiDerm™ tissues started.

3.4.4 MTT-Solution

The MTT-solution was prepared freshly on day of use (resulting: 1 mg/mL).
For use in the pre-test (step 3): MTT from Sigma, Germany, DMEM from Gibco, Germany
For use in the main experiment: MTT concentrate from MatTek, MTT diluent from MatTek.

3.5 Test for Direct MTT Reduction and Colour Interference

A test item may interfere with the MTT endpoint if: a) it is coloured and/or b) able to directly reduce MTT. The MTT assay is affected only if the test item is present in the tissues when the MTT viability test is performed.

Some non-coloured test items may change into coloured test items in wet or aqueous conditions and thus stain tissues during the 60 min exposure. Therefore, before exposure, a functional check for this possibility has to be performed (Step 1).

Step 1
30 µL of the test item were added to 0.3 ml of deionised water. The mixture was incubated in the incubator (37 ± 1.5 °C, 5 ± 0.5 % CO_2) for 60 min. At the end of the exposure time, the mixture was shaken and the presence and intensity of the staining (if any) was evaluated.

Since the test item did not dye water, an additional test with viable tissues (but without MTT addition) was not necessary to be performed.

Step 3

All test items (including those already evaluated in Step 1) are further evaluated for their potential to interfere with MTT assay. To test if an item directly reduces MTT, 30 µL of the test item were added to 1 ml of the MTT-solution (1mg/mL) and was incubated in the incubator (37 ± 1.5 °C, 5 ± 0.5% CO_2) for 60 minutes. Untreated MTT medium was used as control.

Since the MTT solution did not turn blue/purple, the test item was not considered to reduce MTT and an additional test with freeze-killed tissues did not have to be performed.

3.6 Experimental Performance

3.6.1 Pre-warming of EpiDerm™ Tissues

The plastic bag containing the 24-well plate with epidermal tissues was opened under sterile conditions. Under an airflow using forceps, the gauze was removed and the inserts were taken out. Any remaining agarose that adheres to the outer sides of the inserts was removed by gentle blotting on the sterile filter paper or gauze, and the tissues were placed in the empty, sterile 6-well plate. Prior to the exposure of the test item and of the controls the EpiDerm™ tissues were inspected for quality: If necessary, it was taken care, that

1. air bubbles between agarose and insert were not > 30% of the total surface,
2. liquid on top of the insert was removed with sterile cotton tips,
3. if again moisture is observed on top of the inserts after the pre-incubation or in case of visible defects the respective skin models were discarded.

0.9 mL of the assay medium (20 – 25 °C) was pipetted into each well of sterile 6-well plates. The inserts with the EpiDerm™ tissues were placed in the upper wells, and were pre-incubated for 60 minutes in the incubator (37 ± 1.5 °C, 5 ± 1% CO_2, 95 ± 5% RH). Following, the inserts were transferred from upper wells into the lower wells of the 6-well plates, and, the pre-incubation was continued for about further 20 hours (37 ± 1.5 °C, 5 ± 1% CO_2, 95 ± 5% RH).

3.6.2 Treatment

After pre-incubation of EpiDerm™ tissues was completed, medium was replaced by 0.9 mL of fresh medium per well. The negative and positive control, the vehicle control, and the test item were added into the insert atop the corresponding EpiDerm™ triplicate tissues. The treatment time was 60 minutes in total. Within this period the 6-well plates were put into the incubator for 35 minutes at 37 ± 1.5 °C, 5 ± 0.5 % CO_2. In the remaining period the plates were placed in a sterile bench at room temperature until the end of treatment.

After the end of the treatment interval the inserts were removed immediately from the 6-well plate and tissues were gently rinsed with DPBS at least 15 times in order to remove any residual test material. After the rinsing the inserts were submerged in DPBS at least three times. Afterwards the inserts were once again rinsed with sterile DPBS from the inside and

the outside. Excess DPBS was removed by gently shaking the inserts and blotting the bottom with sterile blotting paper. The tissues were carefully dried using sterile cotton tipped swap. The tissues were then transferred into new 6-well plates with 0.9 mL of fresh assay medium in the upper row. The inserts were placed in the prepared holding plate. Tissues were incubated for about 24 hours at 37 ± 1.5 °C, 5 ± 0.5 % CO_2. After incubation the inserts were transferred into new 6-wells plates containing fresh medium. Thereafter tissues were incubated for another 19 hours at 37 ± 1.5 °C, 5 ± 0.5 % CO_2. The complete incubation time was approximately 43 hours.

3.6.3 MTT Assay

On the day of testing the MTT concentrate was diluted with the MTT diluent (1 mg/mL). The 24-well plates were prepared before the end of the tissue pre-warming period. A volume of 300 µL of the MTT solution was added to each well and the plates were kept in an incubator (37 ± 1 °C, 5 ± 0.5 % CO_2) until further use.

After the 42-hours incubation period was completed for all tissues and exposure groups, culture inserts were transferred from the holding plates to the MTT-plates. After a 3-hour incubation period (37 ± 1 °C, 5 ± 0.5 % CO_2), the MTT solution was aspirated from the wells, and the wells were rinsed three times with DPBS. Inserts were transferred onto new 24-well plates. The inserts were immersed into extractant solution by gently pipetting 2 mL extractant solution (isopropanol) in each insert. The level rose above the upper edge of the insert, thus tissues were completely covered from both sides. The 24-well plate was sealed to inhibit the isopropanol evaporation.

The formazan salt was extracted for about 69 hours without shaking in the refrigerator.

After the extraction period was completed, the inserts were pierced with an injection needle to allow the extract to run into the well from which the insert was taken. The 24-well plates were placed on a shaker for 15 minutes until the solution was homogeneous in colour. Afterwards the insert was discarded.

Per each tissue, 3 × 200 µL aliquots of the blue formazan solution were transferred into a 96-well flat bottom microtiter plate from the 15 minutes exposure. OD was read in a microplate reader (Versamax® Molecular Devices, Softmax Pro, version 4.7.1) with a 570 nm filter. Mean values were calculated from the 3 wells per tissue.

3.7 Data Recording

The data generated were recorded in the laboratory protocol. The results are presented in tabular form, including experimental groups with the test item, negative, and positive controls.

3.8 Data Evaluation

The mean OD of the three negative control tissues was calculated after blank correction. This value corresponds to 100% tissue viability in the current test. For each individual tissue treated with the test item or the positive control the individual relative tissue viability is calculated according to the following formula:

$$\text{Relative viability}\,(\%) = \left[\frac{\text{mean OD}_{\text{test item / positive control}}}{\text{mean OD}_{\text{negative control}}}\right] \bullet \quad 100$$

For the test item and the positive control the mean relative viability ± rel. standard deviation of the three individual tissues was calculated and used for classification according to the following prediction model:

For the current test, an irritation potential of the test item of H315, GHS Cat 2 according to UN GHS (published 2003, last (6th) revision 2015) is recommended if the mean relative tissue viability of three individual tissues is reduced ≤ 50% of the negative control.

in vitro result	*in vivo* prediction
mean tissue viability ≤ 50%	irritant (I), H315 (category 2)
mean tissue viability > 50%	non-irritant (NI)

3.8.1 Acceptability of the Assay

Criterion 1 (negative control): The absolute OD 570 nm of the negative control tissues in the MTT test is an indicator of tissue viability obtained after the shipping and storing procedure and under specific conditions of the assay. Tissue viability is meeting the acceptance criterion if the mean OD_{570} of the negative control tissues is ≥ 0.8 and ≤ 2.8.

Criterion 2 (positive control): An assay is meeting the acceptance criterion if mean relative tissue viability of the positive control is ≤ 20%.

Criterion 3 (standard deviation): The SD of 3 identical replicates should be < 18%.

OD values should not be below historically established boundaries.

Historical data (see annex 1) and the quality certificate of the supplier of the test kit (see annex 2) demonstrated the robustness of the test system or rather of the test kit.

4 DEVIATIONS FROM STUDY PLAN

There was the following deviation from the study plan:

Instead of the MatTek EPI-200-SIT Kit, the MatTek EPI-200-PHO Test kit was used for the experimental performance; this Deviation occurred because of changes in the ordering of the tissues.

This Deviation had no influence on the outcome of the study since the tissues in the test kits are identical, with only the cell culture medium being different between the test kits. Because the tissue supplier was informed in advance, he included the required DMEM based assay medium in the shipment.

5 ARCHIVING

Records and documentation relating to this study will be maintained in the archives of Envigo CRS GmbH for a period of 4 years from the date on which the Study Director signs the final report. This will include but may not be limited to the Study Plan, raw data, Report and a sample of the test item.

At termination of the aforementioned period, the records and documentation will be transferred to the GLP compliant Archive Envigo CRS (Switzerland) Limited for further archiving up to a total archiving period of 15 years.

Samples and specimens that no longer afford evaluation will be discarded in accordance with Standard Operating Procedures and without further notice.

Envigo will retain in its archive the study plan and final report, and any amendments indefinitely.

6 RESULTS AND DISCUSSION

6.1 Results

Results after treatment with MIXTURE OF 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-, (3AR,4R,5S,7R,7AR)-REL- AND 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-,(3AR,4S,5R,7S,7AR)-REL- and the controls

Dose Group	Ex-po-sure Interval	Tissue No.	Absor-bance 570 nm Well 1	Absor-bance 570 nm Well 2	Absor-bance 570 nm Well 3	Mean Absor-bance of 3 Wells	Mean-Absorbance of three wells blank corrected	Mean Absor-bance of 3 tissues after blank correction *	Rel. Absor-bance [%] Tissue 1, 2 + 3*	Relative Standard Deviation [%]	Mean Rel. Absorbance [% of Negative Control]***
Blank			0.038	0.039	0.039	0.038	0.000				
Negative Control	60 min	Tissue 1	1.658	1.633	1.664	1.652	1.613	1.690	95.5	4.0	100.0
		Tissue 2	1.792	1.783	1.755	1.777	1.738		102.9		
		Tissue 3	1.782	1.766	1.721	1.756	1.718		101.7		
Positive Control	60 min	Tissue 1	0.104	0.100	0.101	0.101	0.063	0.060	3.7	5.3	3.5
		Tissue 2	0.094	0.096	0.096	0.095	0.057		3.4		
		Tissue 3	0.097	0.098	0.097	0.097	0.059		3.5		
Test Item	60 min	Tissue 1	0.513	0.491	0.495	0.500	0.461	0.472	27.3	5.7	27.9
		Tissue 2	0.480	0.502	0.491	0.491	0.453		26.8		
		Tissue 3	0.496	0.660	0.468	0.541	0.503		29.8		

* relative absorbance per tissue [rounded values]: $$\frac{100 \times (absorbance_{tissue})}{(mean\,absorbance_{negative\ control})}$$

** relative absorbance per treatment group [rounded values]: $$\frac{100 \times (mean\ \ absorbance_{test\,item\,/\,positive\,control})}{(mean\ \ absorbance_{negative\ \ control})}$$

The optical pre-experiment (colour interference pre-experiment) to investigate the test item's colour change potential in water did not led to a change in colour.

Optical evaluation of the MTT-reducing capacity of the test item after 1 hour incubation with MTT-reagent did not show blue colour.

The mean relative absorbance value of the test item, corresponding to the cell viability, decreased to 27.9% (threshold for irritancy: ≤ 50%), consequently the test item was irritant to skin.

6.2 Discussion

This *in vitro* study was performed to assess the irritation potential of MIXTURE OF 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-, (3AR,4R,5S,7R,7AR)-REL- AND 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-,(3AR,4S,5R,7S,7AR)-REL- by means of the Human Skin Model Test.

The test item passed the MTT- and the Colour Interference pre-tests.

30 µL of the test item were applied to each tissue, and spread to match the surface of triplicate tissue.

30 µL of either the negative control (DPBS) or the positive control (5% SLS) were applied to triplicate tissue each.

The test item and the positive and negative controls were washed off the skin tissues after 60 minutes treatment. After further incubation for about 43 hours the tissues were treated with the MTT solution for 3 hours following 69 hours extraction of the colorant from the cells. The amount of extracted colorant was determined photometrically at 570 nm.

After treatment with the negative control the absorbance values were well within the required acceptability criterion of mean OD ≥ 0.8 and ≤ 2.8 for the 60 minutes treatment interval thus showing the quality of the tissues.

Treatment with the positive control induced a decrease in the relative absorbance as compared to the negative control to 3.5% thus ensuring the validity of the test system.

The relative standard deviations between the % variability values of the test item, the positive and negative controls in the main test were below 6% (threshold of the "OECD Guideline for the Testing of Chemicals 439: *In vitro* Skin Irritation: Reconstructed Human Epidermis Test Method": < 18%), thus ensuring the validity of the study.

Compared to the relative absorbance value of the negative control the mean relative absorbance value was reduced to 27.9% after exposure of the skin tissues to the test item. This value is under the threshold for irritancy of $\leq 50\%$. Therefore, the test item is considered to possess an irritant potential.

7 CONCLUSION

In conclusion, it can be stated that in this study and under the experimental conditions reported, MIXTURE OF 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-, (3AR,4R,5S,7R,7AR)-REL- AND 4,7-METHANO-1H-INDENE, 5-ETHOXYOCTAHYDRO-,(3AR,4S,5R,7S,7AR)-REL- is **irritant** to skin according to UN GHS and EU CLP regulation.

8 REFERENCES

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OECD 2010, OECD Guideline for the Testing of Chemicals 439: *in vitro* Skin Irritation: Reconstructed Human Epidermis Test Method (Original Guideline adopted July 28, 2015)

EU 2008 Council Regulation No 440/2008 of 30 May 2008 laying down test methods pursuant to EC Regulation No 1907/2006 of the European Parliament and of the Council on REACH; 1st adaptation to technical progress (ATP) 2009: EC Regulation No 761/2009 of 23 July 2009 amending, for the purpose of its adaptation to technical progress, EC Regulation No 440/2008 laying down test methods pursuant to EC Regulation No 1907/2006 of the European Parliament and of the Council on REACH.

MatTek Corporation 2010, Protocol for: In Vitro EpiDerm™ Skin Irritation Test (EPI-200-SIT) for use with MatTek Corporation's Reconstructed Human Epidermal Model EpiDerm (EPI-200), Rev. 29/06/15.

ANNEXES

Annex 1 Historical Data

Positive Control		Negative Control [OD_{570}]	
Mean Viability	4.64%	**Mean Absorption**	1.74
Rel. Standard Deviation	11.2%	**Rel. Standard Deviation**	8.68%
Range of Viabilities	4.00% - 5.90%	**Range of Absorbance**	1.48 – 1.98
Mean Absorption	0.0803		
Rel. Standard Deviation	12.6%		
Range of Absorbance	0.066 - 0.097		

Data of 31 studies performed from July 2015 until March 2016

Annex 2 Test Kit Certificate

Certificate of Analysis

MatTek | IN VITRO LIFE SCIENCE LABORATORIES

Product: EpiDerm™ Reconstructed Human Epidermis

Lot Number: 23349

Part#: EPI-200, EPI-212, EPI-218

Description: Reconstructed human epidermis tissue containing normal human keratinocytes. This product is for research use only. Not for use in animals, humans or diagnostic purposes.

I. Cell source

All cells used to produce EpiDerm™ are purchased or derived from tissue obtained by MatTek Corporation from accredited institutions. In all cases, consent was obtained by these institutions from the donor or the donor's legal next of kin, for use of the tissues or derivatives of the tissue for research purposes.

Keratinocyte Strain: 00267

II. Analysis for potential biological contaminants

The cells used to produce EpiDerm™ tissue are screened for potential biological contaminants. Tests for each potential biological contaminant listed below were performed according to the test method given. Results of "Not detected" indicate that testing for the potential biological contaminant was not observed as determined by the stated test method.

HIV-1 virus – Oligonucleotide-directed amplification	Not detected
Hepatitis B virus – Oligonucleotide- directed amplification	Not detected
Hepatitis C virus – Oligonucleotide- directed amplification	Not detected
Bacteria, yeast, and other fungi – long term antibiotic, antimycotic free culture	Not detected

III. Analysis for tissue functionality and quality

Test	Specification	Acceptance criteria	Result and QA Statement	
Tissue viability	MTT QC assay, 4 hours, n=3	OD (540-570 nm) [1.0-3.0]	2.141 ± 0.124	Pass
Barrier function	ET-50 assay, 100 µL 1% Triton X-100, 4 time-points, n=3, MTT assay	ET-50 [4.77-8.72 hrs]	6.16 hrs	Pass
Sterility	Long term antibiotic and antimycotic free culture	No contamination	Sterile	Pass

Tissue viability and the barrier function test are within the acceptable ranges and indicate appropriate formation of the epidermal barrier, the presence of a functional stratum corneum, a viable basal cell layer, and intermediate spinous and granular layers. Results obtained with this lot conform to the requirements of the **OECD TG 431 and 439.**

Initials: JL

Date: 10.08.2016

Paul Kearney

Paul Kearney
Quality Assurance Manager

August 10, 2016
Date

CAUTION: Whereas all information herein is believed to be correct, no absolute guarantee that human derived material is non-infectious can be made or is implied by this certificate of analysis. All tissues should be treated as potential pathogens. The use of protective clothing and eyeware and appropriate disposal procedures are strongly recommended.

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Annex 3 Certificate of Analysis

IDENTITY-CERTIFICATE

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Test-Code: PI 1525

Cas-No. : 1897392-68-5 and 13213-08-6

Chemical Name: Mixture of 4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4R,5S,7R,7aR)-rel- and 4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4S,5R,7S,7aR)-rel-

Batch-No.: Ho 154 262 MM + 0.1% Vit.E.

Appearance/colour: Liquid/clear

Expiry date: 2017 Dec.

Purity: 95.3%
Constituent 1: 4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4R,5S,7R,7aR)-rel-, CAS: 1897392-68-5, ca. 83%
Constituent 2: 4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4S,5R,7S,7aR)-rel-, CAS: 13213-08-6, ca. 12%

Storage conditions: Store in a tightly closed container at room temperature away from light and moisture.

Annex 4 GLP Certificate

HESSEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

ENVIGO CRS GmbH
In den Leppsteinswiesen 19
64380 Roßdorf

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according chemVwV-GLP Nr. 5.3/OECD guidance)

2 Prüfungen zur Bestimmung der toxikologischen Eigenschaften	**2** Toxicity studies
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)	**3** Mutagenicity studies
8 Analytische Prüfungen an biologischen Materialien	**8** Analytical studies on biological materials

13. – 16. Juli 2015
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP- Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag

Th. Zimmermann, Referatsleiter, Wiesbaden, den **14. September 2015**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, KLIMASCHUTZ, LANDWIRTSCHAFT UND VERBRAUCHERSCHUTZ

Hess. Ministerium für Umwelt, Klimaschutz, Landwirtschaft und Verbraucherschutz,
Mainzer Straße 80 D65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority